



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 7/10, A61K 37/02		A1	(11) International Publication Number: WO 93/10146
			(43) International Publication Date: 27 May 1993 (27.05.93)
(21) International Application Number: PCT/US92/09842		(74) Agents: DUFT, Bradford, J. et al.; Lyon & Lyon, 611 West Sixth Street, 34th Floor, Los Angeles, CA 90017 (US):	
(22) International Filing Date: 19 November 1992 (19.11.92)			
(30) Priority data: 794,266 19 November 1991 (19.11.91) US		(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).	
(71) Applicant: AMYLIN PHARMACEUTICALS, INC. [US/US]; 9373 Towne Centre Drive, San Diego, CA 92121 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: GAETA, Laura, S., L. ; 3226 Rim Rock Circle, Olivenhain, CA 92024 (US). JONES, Howard ; 16870 St. James Drive, Poway, CA 92064 (US). ALBRECHT, Elisabeth ; 10540 Bannister Way, San Diego, CA 92126 (US).			
(54) Title: AMYLIN AGONIST PEPTIDES AND USES THEREFOR			
<p style="text-align: center;"> ¹Lys-Cys-Asn-Thr-⁵Ala-Thr-Cys-Ala-Thr-¹⁰Gln-Arg-Leu-Ala- Asn-¹⁵Phe-Leu-Val-His-Ser-²⁰Ser-Asn-Asn-Phe-Gly-²⁵Ala- lle-Leu-Ser-Ser-³⁰Thr-Asn-Val-Gly-Ser-³⁵Asn-Thr-Tyr-NH₂ </p>			
(57) Abstract			
<p>Agonist analogues of amylin and related pharmaceutical compositions, and methods of treatment of diabetes and other insulin-requiring states, as well as methods of treatment of hypoglycemia, are provided.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MI	Moldova	VN	Viet Nam
FI	Finland	MO	Mongolia		

DESCRIPTIONAmylin Agonist Peptides And Uses ThereforBackground

This application is a continuation-in-part of U.S. Application Serial No. 07/667,040 filed March 8, 1991, which is hereby incorporated by reference.

5 Field Of The Invention

The field of the invention is medicine, particularly the treatment and prevention of hypoglycemic conditions and other conditions in which enhanced amylin action is of benefit, including insulin-requiring states such as
10 diabetes mellitus. More specifically, the invention relates to the preparation and use of agonist analogues of the peptide hormone amylin.

Description Of Related Art And Introduction To The Invention

15 Diabetes mellitus is a serious metabolic disease that is defined by the presence of chronically elevated levels of blood glucose (hyperglycemia). This state of hyperglycemia is the result of a relative or absolute lack of activity of the peptide hormone, insulin. Insulin is
20 produced and secreted by the β cells of the pancreas. Insulin is reported to promote glucose utilization, protein synthesis, and the formation and storage of neutral lipids. Glucose, the principal source of carbohydrate energy, is stored in the body as glycogen, a
25 form of polymerized glucose, which may be converted back into glucose to meet metabolism requirements. Under normal conditions, insulin is secreted at both a basal rate and at enhanced rates following glucose stimulation, all to maintain metabolic homeostasis by the conversion of
30 glucose into glycogen.

The term diabetes mellitus encompasses several different hyperglycemic states. These states include Type 1 (insulin-dependent diabetes mellitus or IDDM) and Type 2 (non-insulin-dependent diabetes mellitus or NIDDM) diabetes. The hyperglycemia present in individuals with Type I diabetes is associated with deficient, reduced, or nonexistent levels of insulin which are insufficient to maintain blood glucose levels within the physiological range. Treatment of Type 1 diabetes involves administration of replacement doses of insulin, generally by the parenteral route. The hyperglycemia present in individuals with Type II diabetes is initially associated with normal or elevated levels of insulin; however, these individuals are unable to maintain metabolic homeostasis due to a state of insulin resistance in peripheral tissues and liver and, as the disease advances, due to a progressive deterioration of the pancreatic β cells which are responsible for the secretion of insulin. Thus, initial therapy of Type 2 diabetes may be based on diet and lifestyle changes augmented by therapy with oral hypoglycemic agents such as sulfonylureas. Insulin therapy is often required, however, especially in the latter stages of the disease, in attempting to produce some control of hyperglycemia and minimize complications of the disease. Thus, many Type 2 diabetics ultimately require insulin in order to survive.

Amyloid is the name given to extracellular deposits of β sheet protein filaments. Deposits of amyloid material have been reported to be found in pancreas of patients with Type 2 diabetes mellitus. Other studies have indicated that the degree of amyloid depositions increases with the degree of hyperglycemia in humans and the severity of Type 2 diabetes. Chemical analysis of pancreatic amyloid led to the surprising and unexpected discovery of the peptide hormone, amylin. Clark, A., et al., Lancet ii: 231-234 (1987). This peptide was discovered to be comprised of 37 amino acids, none of

which are acidic residues, to have a disulfide linkage between the cysteine residues at positions 2 and 7, and to be C-terminally amidated. Amylin is the major protein constituent of the amyloid which is reported to be found
5 in the pancreatic Islets of Langerhans in patients with type 2 diabetes mellitus.

It has been reported that the presence of both the intramolecular cystine bridge and the carboxy terminal amide group in the peptide structure of the synthetic
10 molecule yield the greatest biological activity to inhibit glycogen synthesis in skeletal muscle. E.g., Cooper, G.J.S., et al., Proc. Natl. Acad. Sci. (USA) 84:8628-8632 (1987); Cooper G.J.S., et al., in Diabetes 1988, ed. Larkins, R., Zimmet, P. & Chisholm, D. (Elsevier, Amsterdam), pp. 493-496 (1989). The amino acid sequence
15 of amylin (see Figure 1) has 46% homology with human calcitonin gene related peptide 2 (CGRP-2).

One report states that a limited segment of the amylin molecule, residues 20-29, is a potential
20 contributor toward amyloid fibril formation in the islets of Langerhans in Type 2 diabetes mellitus. Glenner et al., Biochem. Biophys. Res Commun. 155:608-614 (1988). It has also been reported that amino acid sequence differences between amylin from certain mammalian species
25 occur in this region, and further investigation has focused on identifying residues linked to amyloid formation. Westermarck et al., Proc. Natl. Acad. Sci. (USA) 87: 5036-5040 (1990). The study of Westermarck et al. reported attempts to synthesize various 20-29 amino
30 acid segments of amylin sequences from different species followed by a comparison of their ability to form amyloid fibrils. It was proposed that the residues 25-29 of human amylin were the most strongly amyloidogenic and that the proline-for-serine substitution in position 28, as in
35 several rodent species, significantly inhibited fibril formation in the studied decapeptides.

Amylin is a complex peptide, and the synthesis of bioactive preparations of amylin is laborious. Amylin has also been found to have limited solubility and limited stability in solution. We have found that rat amylin has a higher solubility and stability in solution than human amylin. This may be due in some measure, although this is not known, to the different aggregation properties of the amylin from different species. Only the human, non-human primate, and cat species of amylin have been reported to aggregate to form islet amyloid in vivo. The sequences of amylin now reported to have been isolated from a number of species are set forth in Figure 2.

In Type I diabetes, amylin levels are severely reduced or are nonexistent when compared to normal controls. In the disease state of Type I diabetes mellitus, the β -cells, which are the producers of insulin and amylin, have been destroyed by an autoimmune process. Amylin has been proposed to be useful in the treatment of diabetes mellitus and hypoglycemia, including insulin-induced hypoglycemia. It has also been proposed that the co-administration of insulin with amylin is a superior therapy to the existing administration of insulin alone, and that coadministration of amylin with glucagon for the treatment of hypoglycemia is a superior therapy to the existing administration of glucagon alone. It would be useful to provide, for such purposes and others, less complicated compounds that have the activities of native human amylin, as well as compounds which may show enhanced solubility and/or stability over native human amylin. Such compounds are described and claimed herein.

Summary Of The Invention

The present invention is directed to novel analogues of the peptide hormone amylin. These compounds mimic the effects of amylin, and are referred to as amylin agonists or as agonist analogues of amylin.

The invention is also directed to pharmaceutical compositions comprising the agonist analogues of the present invention, and to methods of treatment and prevention of hypoglycemic conditions and other conditions in which enhanced amylin action is of benefit, including insulin-requiring states such as diabetes mellitus, comprising administering an agonist analogue of amylin to an animal (alone or in conjunction with an insulin or a glucagon).

10 Definitions

As used herein, the following terms have the following meanings unless expressly stated to the contrary:

The term "alkyl" refers to both straight- and branched-chain alkyl groups. The term "lower alkyl" refers to both straight- and branched-chain alkyl groups having a total of from 1 to 6 carbon atoms and includes primary, secondary and tertiary alkyl groups. Typical lower alkyls include, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, t-butyl, n-pentyl, n-hexyl, and the like.

The term "aryl" refers to carbocyclic aromatic groups of 6 to 14 carbon atoms such as phenyl and naphthyl, as well as heterocyclic aromatic groups containing 1 to 3 heteroatoms (nitrogen, oxygen, sulfur, etc.) such as pyridyl, triazolopyrazine, pyrimidine and the like.

The term "aralkyl" refers to an "aryl" group of 6 to 10 carbon atoms directly attached to an "alkyl" group of 1 to 4 carbon atoms and includes for example benzyl, p-chlorobenzyl, p-methylbenzyl, and 2-phenylethyl.

The term "cycloalkyl" refers to cyclic alkyl groups of 5 to 8 carbon atoms.

Brief Description Of The Drawings

FIG. 1 depicts the amino acid sequence of human amylin.

FIG. 2 depicts a comparison of amino acid sequences of amylin isolated from several mammals.

FIG. 3 depicts the amino acid sequence of novel amylin agonist peptides.

5 Detailed Description Of The Invention

According to the present invention, novel agonist analogues of amylin are provided. These analogues are useful as agonists of amylin, including as hyperglycemics, and may be represented by Figure 3.

- 10 In one aspect, the present invention is directed to agonist analogues of Figure 3, wherein A₁ is hydrogen Lys, Ser, Ala, des- α -amino Lys, or acetylated Lys; B₁ is Ala, Ser or Thr; C₁ is Val, Leu or Ile; D₁ is His or Arg; E₁ is Ser or Thr; F₁ is Ser, Thr, Gln or Asn; G₁ is Asn, Gln or
- 15 His; H₁ is Phe, Leu or Tyr; I₁ is Ala or Pro; J₁ is Ile, Val, Ala or Leu; K₁ is Ser, Pro, Leu, Ile or Thr; L₁ is Ser, Pro or Thr; M₁ is Asn, Asp or Gln; X and Y are independently selected residues having side chains which are chemically bonded to each other to form an
- 20 intramolecular linkage; and Z is hydroxy, amino, alkylamino, dialkylamino, cycloalkylamino, arylamino, aralkylamino, alkylloxy, aryloxy or aralkyloxy; provided that (a) when A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is His, E₁ is Ser, F₁ is Ser, G₁ is Asn, H₁ is Phe, I₁ is Ala, J₁ is
- 25 Ile, K₁ is Ser, L₁ is Ser, and M₁ is Asn; (b) when A₁ is Lys, B₁ is Ala, C₁ is Ile, D₁ is Arg, E₁ is Ser, F₁ is Ser, G₁ is Asn, H₁ is Leu, I₁ is Ala, J₁ is Ile, K₁ is Ser, L₁ is Pro, and M₁ is Asn; (c) when A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is Arg, E₁ is Thr, F₁ is Ser, G₁ is Asn, H₁ is Leu,
- 30 I₁ is Ala, J₁ is Ile, K₁ is Ser, L₁ is Pro, and M₁ is Asn; (d) when A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is Arg, E₁ is Ser, F₁ is Ser, G₁ is Asn, H₁ is Leu, I₁ is Pro, J₁ is Val, K₁ is Pro, L₁ is Pro, and M₁ is Asn; (e) when A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is His, E₁ is Ser, F₁ is Asn, G₁ is Asn,
- 35 H₁ is Leu, I₁ is Pro, J₁ is Val, K₁ is Ser, L₁ is Pro and M₁

is Asn; or (f) when A₁ is Lys, B₁ is Thr, C₁ is Val, D₁ is Arg, E₁ is Ser, F₁ is Ser, G₁ is His, H₁ is Leu, I₁ is Ala, J₁ is Ala, K₁ is Leu, L₁ is Pro and M₁ is Asp; then one or more of any of A₁ to M₁ is not an L-amino acid and Z is not
5 amino.

Suitable side chains for X and Y include groups derived from alkyl sulfhydryls which may form disulfide bonds; alkyl acids and alkyl amines which may form cyclic lactams; alkyl aldehydes or alkyl halides and alkylamines
10 which may condense and be reduced to form an alkyl amine bridge; or side chains which may be connected to form an alkyl, alkenyl, alkynyl, ether or thioether bond. Preferred alkyl chains include lower alkyl groups having from about 1 to about 6 carbon atoms.

15 An additional aspect of the present invention is directed to agonist analogues of Figure 3 which are not bridged, and wherein X and Y are independently selected from Ala, Ser, Cys, Val, Leu and Ile or alkyl, aryl, or aralkyl esters and ethers of Ser or Cys.

20 Biologically active derivatives of the above Figure 3 agonist analogues are also included within the scope of this invention in which the stereochemistry of individual amino acids may be inverted from (L)/S to (D)/R at one or more specific sites.

25 Also included within the scope of this invention are the agonist analogues modified by glycosylation of Asn, Ser and/or Thr residues.

Biologically active agonist analogues of amylin are included within the scope of this invention which contain
30 less peptide character. Such peptide mimetics may include, for example, one or more of the following substitutions for -CO-NH-amide bonds: depsipeptides (-CO-O-), iminomethylenes (-CH₂-NH-), trans-alkenes (-CH=CH-), β-enaminonitriles (-C(=CH-CN)-NH-), thioamides (-CS-NH-),
35 thiomethylenes (-S-CH₂- or -CH₂-S-), methylenes (-CH₂-CH₂-) and retro-amides (-NH-CO-).

Compounds of this invention form salts with various inorganic and organic acids and bases. Such salts include salts prepared with organic and inorganic acids, for example, HCl, HBr, H_2SO_4 , H_3PO_4 , trifluoroacetic acid, 5 acetic acid, formic acid, methanesulfonic acid, toluenesulfonic acid, maleic acid, fumaric acid and camphorsulfonic acid. Salts prepared with bases include, for example, ammonium salts, alkali metal salts (such as sodium and potassium salts) and alkali earth salts (such 10 as calcium and magnesium salts). Acetate, hydrochloride, and trifluoroacetate salts are preferred.

The salts may be formed by conventional means, as by reacting the free acid or base forms of the product with one or more equivalents of the appropriate base or acid in 15 a solvent or medium in which the salt is insoluble, or in a solvent such as water which is then removed in vacuo or by freeze-drying or by exchanging the ions of an existing salt for another ion on a suitable ion exchange resin.

The compounds of the invention include various stereoisomers. In the preferred compounds of this invention, the chiral centers on the peptide backbone are all S. 20

Compounds of the present invention may be prepared by using certain conventional coupling reactions known in the peptide art. The analogues of this invention are prepared 25 by successively adding the desired amino acid to a growing peptide chain. Typically, an α -N-carbamoyl protected amino acid and an amino acid attached to the growing peptide chain on a resin support are reacted at room temperature in an inert solvent such as N-methylpyrrolidone, dimethylformamide or methylene chloride in the 30 presence of coupling agents such as dicyclohexylcarbodiimide 1-hydroxybenzotriazole in the presence of a base such as diisopropylethylamine. The α -N-carbamoyl protecting group is removed from the resultant peptide 35 with a reagent such as trifluoroacetic acid or piperidine, and the coupling reaction repeated with the next desired N-protected amino acid. Suitable N-protecting groups are

known in the art, with t-butyloxycarbonyl herein preferred.

Certain preferred methods for synthesis are described in the commonly-assigned copending and commonly assigned patent application Serial No. 667,040 ("Synthetic Preparation of Amylin and Amylin Analogs", filed March 8, 1991). These methods provide for solid phase synthesis of a peptide which comprises amylin or an amylin analog which has enhanced biological activity and is substantially free of deletion and other contaminating peptides wherein said peptide is synthesized using successive synthesis cycles, whereby in each such synthesis cycle, a designated amino acid is added to a growing peptide chain attached to an insoluble resin support by formation of a peptide linkage between an α -amino group of the growing peptide chain and on α -carboxyl of the designated amino acid; and wherein each synthesis cycle comprises: (a) treating the growing peptide chain under α -amino deprotecting conditions to remove an α -amino group; (b) activating the α -carboxyl group of the α -amino protected designated amino acid; (c) contacting the growing peptide chain and the designated amino acid under coupling conditions to form a peptide linkage between the free α -amino for the peptide chain and the activated α -carboxyl of the designated amino acid; and (d) repeating steps (b) and (c) if the coupling efficiency of step (c) is less than about 97%. It is preferred to repeat steps (b) and (c) if the coupling efficiency is less than about 99%. In another preferred aspect, steps (b) and (c) are repeated in each synthesis cycle. Optionally, the coupling efficiency is measured after each coupling step.

Suitable coupling conditions include use of a solvent system which maximizes swelling of the solid support, minimizes secondary structure elements of the peptide chain during synthesis cycles, and minimizes intrapeptide and interpeptide hydrogen bonding. Preferably the synthesis cycle includes a capping step after the coupling

10

step(s) wherein unreacted α -amino groups of the peptide chain are rendered unreactive. The synthesis cycle is successively repeated using appropriate protected α -amino acids to give amylin or an amylin analog of specified sequence. After completions of the successive synthesis cycles, said amylin or amylin analog is cleaved from the solid support. It is preferred that the cysteine residues of the peptide chain are selectively deprotected and an intramolecular disulfide bond is formed before cleaving the peptide bond from the solid support.

Suitable α -amino protective groups include t-butoxycarbonyl and 9-fluorenylmethoxycarbonyl. In one preferred aspect, when t-butoxycarbonyl is used as the α -amino protecting group, the α -carboxyl groups are activated using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole to form 1-hydroxybenzotriazole esters. A particularly preferred solvent system comprise N-methylpyrrolidone.

The preparation of certain agonist analogues of amylin within the invention is described in Examples 1 to 17 herein. In addition, other agonist analogues which may be prepared according to the above procedures are set forth in Table II herein. The compounds of the invention may also be prepared using recombinant DNA techniques, using methods now known in the art. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor (1989).

The nomenclature of the compounds of the present invention can be used to indicate both the peptide that the sequence is based on and the modifications made to any basic peptide amylin sequence, such as human amylin. An amino acid preceded by a superscript number indicates that the named amino acid replaces the amino acid normally present at the amino acid position of the superscript in the basic amino acid sequence. For example, "¹⁸Arg^{25,28}Pro-h-amylin" refers to a peptide based on the sequence of "h-amylin" or "human-amylin" having the following substitutions: Arg replacing His at residue 18, Pro

replacing Ala at residue 25 and Pro replacing Ser at residue 28. The term "des-¹Lys-h-amylin" refers to a peptide based on the sequence of human amylin, with the first, or N-terminal, amino acid deleted.

5 The agonist analogues of amylin of this invention are useful in view of their pharmacological properties. In particular, compounds of this invention possess activity as amylin agonist agents, as will be evidenced by activity in the receptor binding assay and the soleus muscle assay
10 described in Examples 18 and 19, respectively. Amylin agonist activity of compounds may also be assessed by the ability to induce hyperlactemia and/or hyperglycemia in mammals. In addition to the description of compounds pursuant to Figure 3, certain preferred compounds are set
15 forth in Table I. The preferred compounds des-¹Lys-h-amylin, ²⁸Pro-h-amylin, ^{25,28,29}Pro-h-amylin, ¹⁸Arg^{25,28}Pro-h-amylin, and des-¹Lys¹⁸Arg^{25,28}Pro-h-amylin, all show amylin activity in vivo in treated test animals, provoking marked hyperlactemia followed by hyperglycemia. In addition to
20 having activities characteristic of amylin, certain of the preferred compounds of the invention have also been found to possess more desirable solubility and stability characteristics when compared to human amylin. These preferred compounds include ²⁵Pro²⁶Val^{28,29}Pro-h-amylin,
25 ^{25,28,29}Pro-h-amylin, and ¹⁸Arg^{25,28}Pro-h-amylin.

Compounds described herein which are especially preferred include ¹⁸Arg^{25,28}Pro-h-amylin, des-¹Lys ¹⁸Arg^{25,28}Pro-h-amylin, ¹⁸Arg^{25,28,29}Pro-h-amylin, des-¹Lys¹⁸Arg^{25,28,29}Pro-h-amylin, ^{25,28,29}Pro-h-amylin, des-¹Lys^{25,28,29}Pro-h-amylin, and
30 ²⁵Pro²⁶Val^{25,28}Pro-h-amylin. Still further amylin agonist peptide compounds are listed in Table II. They include:

²³Leu²⁵Pro²⁶Val^{28,29}Pro-h-amylin;
²³Leu²⁵Pro²⁶Val²⁸Pro-h-amylin;
des-¹Lys²³Leu²⁵Pro²⁶Val²⁸Pro-h-amylin;
35 ¹⁸Arg²³Leu²⁵Pro²⁶Val²⁸Pro-h-amylin;
¹⁸Arg²³Leu^{25,28,29}Pro-h-amylin;
¹⁸Arg²³Leu^{25,28}Pro-h-amylin;

- ¹⁷Ile²³Leu^{25,28,29}Pro-h-amylin;
¹⁷Ile^{25,28,29}Pro-h-amylin;
des-¹Lys¹⁷Ile²³Leu^{25,28,29}Pro-h-amylin;
¹⁷Ile¹⁸Arg²³Leu-h-amylin;
5 ¹⁷Ile¹⁸Arg²³Leu²⁶Val²⁹Pro-h-amylin;
¹⁷Ile¹⁸Arg²³Leu²⁶Val^{28,29}Pro-h-amylin;
¹³Thr²¹His²³Leu²⁶Ala²⁸Leu²⁹Pro³¹Asp-h-amylin;
¹³Thr²¹His²³Leu²⁶Ala²⁹Pro³¹Asp-h-amylin;
des-¹Lys¹³Thr²¹His²³Leu²⁶Ala²⁸Pro³¹Asp-h-amylin;
10 ¹³Thr¹⁸Arg²¹His²³Leu²⁶Ala²⁹Pro³¹Asp-h-amylin;
¹³Thr¹⁸Arg²¹His²³Leu^{28,29}Pro³¹Asp-h-amylin; and
¹³Thr¹⁸Arg²¹His²³Leu²⁵Pro²⁶Ala^{28,29}Pro³¹Asp-h-amylin;

The compounds of this invention can be combined with pharmaceutical carriers to prepare pharmaceutical forms suitable for parenteral administration. Experimental responses of the compounds support the clinical application of such pharmaceutical compositions in the treatment of diabetes mellitus and other insulin-requiring states, as well as in the prevention and treatment of 20 episodes of hypoglycemia. The compounds of this invention can also be combined with insulin for the treatment of diabetes mellitus and other insulin-requiring states. By "insulin" is meant a polypeptide or its equivalent useful in regulation of blood glucose levels. A general description of such insulins is provided in Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th Ed., Pergamon Press (1990). Such insulins can be fast acting, intermediate acting, or long acting. Various derivatives of insulin exist and are useful in this 30 invention. See, e.g., U.S. Patent Nos. 5,049,547, 5,028,587, and 5,016,643. Insulin peptides are also useful (see, e.g., U.S. Patent No. 5,008,241), as are analogues (see, e.g., U.S. Patent No. 4,992,417 and 4,992,418). Such compositions can be administered by any 35 standard route, including nasal administration (see, e.g., U.S. Patent Nos. 4,988,512 and 4,985,242, and 2 BioWorld Today, No. 125 (1991)). The compounds of this invention

are also useful in combination with a glucagon for the prevention and treatment of hypoglycemia. See Young et al., U.S. Application Serial No. 07/640,478, filed January 10, 1991, entitled "Hyperglycemic Compositions," which is incorporated herein by reference.

Compositions or products of the invention may conveniently be provided in the form of solutions suitable for parenteral (including intravenous, intramuscular and subcutaneous) or nasal or oral administration. In many cases, it will be convenient to provide an agonist analogue of amylin and an insulin or glucagon in a single composition or solution for administration together. In other cases, it may be more advantageous to administer an insulin or a glucagon separately from said agonist analogue. A suitable administration format may best be determined by a medical practitioner for each patient individually. Suitable pharmaceutically acceptable carriers and their formulation are described in standard formulation treatises, e.g., Remington's Pharmaceutical Sciences by E.W. Martin. See also Wang, Y.J. and Hanson, M.A. "Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers," Journal of Parenteral Science and Technology, Technical Report No. 10, Supp. 42:2S (1988). Suitable formulations including insulin or glucagon are known in the art.

The agonist preparations of the invention may be stabilized at neutral pH. Since the products of the invention are amphoteric they may be utilized as free bases, as acid addition salts or as metal salts. The salts must, of course, be pharmaceutically acceptable, and these will include metal salts, particularly alkali and alkaline earth metal salts, e.g., potassium or sodium salts. A wide variety of pharmaceutically acceptable acid addition salts are available, as described above. These include those prepared from both organic and inorganic acids, preferably mineral acids. Typical acids which may be mentioned by way of example include citric, succinic,

lactic, hydrochloric and hydrobromic acids. Such products are readily prepared by procedures well known to those skilled in the art.

The products of the invention will normally be provided as parenteral compositions for injection or infusion. They can, for example, be suspended in an inert oil, suitably a vegetable oil such as sesame, peanut, or olive oil. Alternatively, they can be suspended in an aqueous isotonic buffer solution at a pH of about 5.6 to 7.4. Useful buffers include sodium citrate-citric acid and sodium phosphate-phosphoric acid. A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection.

The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

If desired, solutions of the above compositions may be thickened with a thickening agent such as methyl cellulose. They may be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acacia powder, a non-ionic surfactant (such as a Tween), or an ionic surfactant (such as alkali polyether alcohol sulfates or sulfonates, e.g., a Triton).

The therapeutically useful compositions of the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be simply mixed in a blender or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and

possibly a buffer to control pH or an additional solute to control tonicity.

For use by the physician, the compositions will be provided in dosage unit form containing an amount of an agonist compound with or without insulin or glucagon which will be effective in one or multiple doses to control or reestablish blood sugar at the selected level. Therapeutically effective amounts of an agonist analogue of amylin as described herein for the treatment of hypoglycemia are those that increase blood sugar levels, preferably to above 80 mg/dl. Therapeutically effective amounts of such agonist analogues for the treatment of diabetes mellitus and other insulin-requiring states are those sufficient to provide for reduced incidence of insulin overdose or undesired hypoglycemia. As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age and weight of the patient, the patient's physical condition, the blood sugar level to be obtained, and other factors. Typical dosage units for treatment of diabetes mellitus will contain from about 0.1 to 5 mg of an amylin agonist compound and, if desired, about 0.5 to about 10 mg of an insulin. Typical dosage units for the treatment of hypoglycemia will contain about 0.5 to 1.0 mg of an amylin agonist compound and, if desired, the art recognized quantity, or less, of a glucagon.

As set forth above, compositions useful in the invention are formulated by standard procedure. These compositions are also administered by standard procedure. Suitable doses are readily determined by those in the art, examples of which are provided above.

To assist in understanding the present invention, the following examples are included which describe the results of a series of experiments. The following examples relating to this invention should not, of course, be construed as specifically limiting the invention. Such variations of the invention, now known or later developed,

which would be within the purview of one skilled in the art are considered to fall within the scope of the present invention as hereinafter claimed.

Examples

5 Example 1

Preparation of ²⁸Pro-human-Amylin

Solid phase synthesis of this analogue of human ("h-") amylin using methylbenzhydrylamine anchor-bond resin and N^ε-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ²⁷[disulfide]amylin-MBHA-resin was obtained by treatment of AcM-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were
15 cleaved with liquid hydrofluoric acid ("HF") in the presence of dimethylsulfide and anisole. The ²⁸Pro-h-amylin was purified by preparative HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid
20 analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: (M+1)/e=3914.

Example 2

Preparation of ²⁵Pro²⁶Val^{28,29}Pro-h-Amylin

Solid phase synthesis of this amylin analogue using
25 methylbenzhydrylamine anchor-bond resin and N^ε-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ²⁷-[disulfide]amylin-MBHA-resin was obtained by treatment with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved
30 the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The ²⁵Pro²⁶Val^{28,29}Pro-h-amylin was purified by preparative HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the
35 structure confirmed by amino acid analysis and sequence

analysis. The product gave the desired mass ion. FAB mass spec: (M+1)/e=3936.

Example 3

Preparation of $^{27}\text{Cyclo-}[^2\text{Asp},^7\text{Lys}]\text{-h-Amylin}$

- 5 Solid phase synthesis of this amylin analogue using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. ^2Asp and ^7Lys were introduced with
- 10 Boc- ^2Asp (Fmoc) -OH and Boc- ^7Lys (Fmoc)-OH. Following selective side-chain deprotection with piperidine the side-chain to side-chain ($^2\text{Asp-}^7\text{Lys}$) cyclization was carried out using benzotriazol-1-yl-oxy-tris (dimethyl-amino)-phosphonium hexafluorophosphate (BOP reagent). Cyclization was as described in Di Maio, J., et al.,
- 15 J. Med. Chem. **33**:661-667 (1990); Felix, A.M., et al., Int J. Pent. Prot. Res. **32**:441 (1988). The $^{27}\text{cyclo-}[^2\text{Asp},^7\text{Lys}]$ amylin-MBHA-resin obtained after cyclization was cleaved with liquid HF in the presence of dimethylsulfide and anisole. The $^{27}\text{cyclo-}[^2\text{Asp},^7\text{Lys}]\text{-h-amylin}$ was purified by
- 20 preparative HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. FAB mass spec: (M+1)/e=3925.

Example 4

Preparation of des- ^1Lys -h-Amylin

- 25 Solid phase synthesis of des- ^1Lys -h-amylin (also represented as $^{27}\text{h-amylin}$) using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods.
- 30 The $^{27}\text{-[disulfide]amylin-MBHA-resin}$ was obtained by treatment of Acn-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of
- 35 dimethylsulfide and anisole. The des- ^1Lys -h-amylin was

purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product
5 gave the desired mass ion. FAB mass spec: $(M+H)^+ = 3,775$.

Example 5

Preparation of 'Ala-h-Amylin

Solid phase synthesis of 'Ala-h-amylin using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-
10 side chain protection was carried out by standard peptide synthesis methods. The 2,7-[disulfide]amylin-MBHA-resin was obtained by treatment of Ac⁶-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain
15 protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The 'Ala-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by
20 amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: $(M+H)^+ = 3,847$.

Example 6

Preparation of 'Ser-h-Amylin

Solid phase synthesis of 'Ser-h-amylin using methyl-
25 benzhydrylamine anchor-bond resin and N⁶-Boc/benzylside chain protection was carried out by standard peptide synthesis methods. The 2,7-[disulfide]amylin-MBHA-resin was obtained by treatment of Ac⁶-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain
30 protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The 'Ser-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by
35 capillary electrophoresis and the structure confirmed by

amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: $(M+H)^+ = 3,863$.

Example 7

Preparation of 29 Pro-h-Amylin

5 Solid phase synthesis of this analogue of human amylin using methylbenzhydrylamine anchor-bond resin and N³-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The 27 -(disulfide) amylin-MBHA-resin was obtained by treatment of Acm-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The 29 Pro-h-amylin was purified by preparative
10 HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: $(M+H)^+ = 3916$.

Example 8

Preparation of 25,28 Pro-h-Amylin

Solid phase synthesis of 25,28 Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and Na-Boc/benzyl-side chain protection was carried out by standard peptide
25 synthesis methods. The 27 -(disulfide) amylin-MBHA-resin was obtained by treatment of Acm-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The 25,28 Pro-h-amylin was purified by preparative reversed-phase HPLC.
30 The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: $(M+H)^+ = 3,939$.

Example 9Preparation of des-¹Lys^{25,28}Pro-h-Amylin

Solid phase synthesis of des-¹Lys^{25,28}Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-
side chain protection was carried out by standard peptide
synthesis methods. The 2,7-[disulfide]amylin-MBHA-resin was
obtained by treatment of Acn-protected cysteines with
thallium (III) trifluoroacetate in trifluoroacetic acid.
After cyclization was achieved the resin and side chain
protecting groups were cleaved with liquid HF in the
presence of dimethylsulfide and anisole. The des-¹Lys^{25,28}Pro-
h-amylin was purified by preparative reversed-phase HPLC.
The peptide was found to be homogeneous by analytical HPLC
and capillary electrophoresis and the structure confirmed
by amino acid analysis and sequence analysis. The product
gave the desired mass ion. FAB mass spec: (M+H)⁺=3,811.

Example 10Preparation of ¹⁸Arg^{25,28}Pro-h-Amylin

Solid phase synthesis of ¹⁸Arg^{25,28}Pro-h-amylin using
methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-
side chain protection was carried out by standard peptide
synthesis methods. The 2,7-[disulfide]amylin-MBHA-resin was
obtained by treatment of Acn-protected cysteines with
thallium (III) trifluoroacetate in trifluoroacetic acid.
After cyclization was achieved the resin and side chain
protecting groups were cleaved with liquid HF in the
presence of dimethylsulfide and anisole. The ¹⁸Arg^{25,28}Pro-h-
amylin was purified by preparative reversed-phase HPLC.
The peptide was found to be homogeneous by analytical HPLC
and capillary electrophoresis and the structure confirmed
by amino acid analysis and sequence analysis. The product
gave the desired mass ion. FAB mass spec: (M+H)⁺=3,959.

Example 11Preparation of des-¹Lys¹⁸Arg^{25,28}Pro-h-Amylin

Solid phase synthesis of des-¹Lys¹⁸Arg^{25,28}Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and N^ε-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ^{2,7}-[disulfide] amylin-MBHA-resin was obtained by treatment of Acm-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The des-¹Lys¹⁸Arg^{25,28}Pro-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: (M+H)⁺=3,832.

Example 12Preparation of ¹⁸Arg^{25,28,29}Pro-h-Amylin

Solid phase synthesis of ¹⁸Arg^{25,28,29}Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and N^ε-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ^{2,7}-[disulfide] amylin-MBHA-resin was obtained by treatment of Acm-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The ¹⁸Arg^{25,28,29}Pro-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: (M+H)⁺3,971.

Example 13Preparation of des-¹Lys¹⁸Arg^{25,28,29}Pro-h-Amylin

Solid phase synthesis of des-¹Lys¹⁸Arg^{25,28,29}Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and N³-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ^{2,7}-[disulfide] amylin-MBHA-resin was obtained by treatment of Ac-m-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The des-¹Lys¹⁸Arg^{25,28,29}Pro-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: (M+H)⁺=3,843.

Example 14Preparation of ^{25,28,29}Pro-h-Amylin

Solid phase synthesis of ^{25,28,29}Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and N³-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ^{2,7}-[disulfide] amylin-MBHA-resin was obtained by treatment of Ac-m-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The ^{25,28,29}Pro-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: (M+H)⁺=3,949.

Example 15Preparation of des-¹Lys^{25,28,29}Pro-h-Amylin

Solid phase synthesis of des-¹Lys^{25,28,29}Pro-h-amylin using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-side chain protection was carried out by standard peptide synthesis methods. The ²⁷-[disulfide] amylin-MBHA-resin was obtained by treatment of Ac-m-protected cysteines with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization was achieved in the resin and side chain protecting groups were cleaved with liquid HF in the presence of dimethylsulfide and anisole. The des-¹Lys^{25,28,29}Pro-h-amylin was purified by preparative reversed-phase HPLC. The peptide was found to be homogeneous by analytical HPLC and capillary electrophoresis and the structure confirmed by amino acid analysis and sequence analysis. The product gave the desired mass ion. FAB mass spec: (M+H)⁺=3,823.

Example 16Preparation of des-¹Lys²⁵Pro²⁶Val^{28,29}Pro-h-Amylin

Solid phase synthesis of this h-amylin analogue using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-side chain protection is carried out by standard peptide synthesis methods, and the ²⁷-[disulfide] amylin-MBHA-resin obtained by treatment with thallium (III) trifluoroacetate in trifluoroacetic acid. After cyclization is achieved the resin and side chain protecting groups are cleaved with liquid HF in the presence of dimethylsulfide and anisole. The des-¹Lys²⁵Pro²⁶Val^{28,29}Pro-h-amylin is then purified by preparative HPLC.

Example 17Preparation of (D)-¹¹Arg1-Amylin

Solid phase synthesis of this amylin analogue using methylbenzhydrylamine anchor-bond resin and N⁶-Boc/benzyl-side chain protection is carried out by standard peptide synthesis methods. (D)-¹¹Arg is introduced with Boc-

(D)-¹¹Arg(Mtr)-OH. The ^{2,7}-[disulfide]amylin-MBHA-resin, obtained by treatment with thallium (III) trifluoroacetate in trifluoroacetic acid, is cyclized and the resin and side chain protecting groups are cleaved with liquid HF in the presence of dimethylsulfide and anisole. The [(D)-¹¹Arg]-amylin is then purified by preparative HPLC.

Example 18

Receptor Binding Assay

Evaluation of the binding of compounds of the invention to amylin receptors was carried out as follows. ¹²⁵I-rat amylin (Bolton-Hunter labeled at the N-terminal lysine) was purchased from Amersham Corporation (Arlington Heights, IL). Specific activities at time of use ranged from 1950 to 2000 Ci/mmol. Unlabeled peptides were obtained from BACHEM Inc. (Torrance, CA) and Peninsula Laboratories (Belmont, CA).

Male Sprague-Dawley rats (200-250) grams were sacrificed by decapitation. Brains were removed to cold phosphate-buffered saline (PBS). From the ventral surface, cuts were made rostral to the hypothalamus, bounded laterally by the olfactory tracts and extending at a 45° angle medially from these tracts. This basal forebrain tissue, containing the nucleus accumbens and surrounding regions, was weighed and homogenized in ice-cold 20 mM HEPES buffer (20 mM HEPES acid, pH adjusted to 7.4 with NaOH at 23°C). Membranes were washed three times in fresh buffer by centrifugation for 15 minutes at 48,000 x g. The final membrane pellet was resuspended in 20 mM HEPES buffer containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF).

To measure ¹²⁵I-amylin binding, membranes from 4 mg original wet weight of tissue were incubated with ¹²⁵I-amylin at 12-16 pM in 20 mM HEPES buffer containing 0.5 mg/ml bacitracin, 0.5 mg/ml bovine serum albumin, and 0.2 mM PMSF. Solutions were incubated for 60 minutes at 23°C. Incubations were terminated by filtration through GF/B

glass fiber filters (Whatman Inc., Clifton, NJ) which had been presoaked for 4 hours in 0.3% polyethyleneimine in order to reduce nonspecific binding of radiolabeled peptides. Filters were washed immediately before
5 filtration with 5 ml cold PBS, and immediately after filtration with 15 ml cold PBS. Filters were removed and radioactivity assessed in a gamma-counter at a counting efficiency of 77%. Competition curves were generated by measuring binding in the presence of 10^{-12} to 10^{-6} M
10 unlabeled test compound and were analyzed by nonlinear regression using a 4-parameter logistic equation (Inplot program; GraphPAD Software, San Diego).

In this assay, purified human amylin binds to its receptor at a measured IC_{50} of about 50 pM. Results for
15 test compounds of the invention are set forth in Table I, showing that each of the compounds has significant receptor binding activity.

Example 19

Soleus Muscle Assay

20 Evaluation of the amylin agonist activity of compounds of the invention was carried out using the soleus muscle assay as follows. Male Harlan Sprague-Dawley rats of approximately 200g mass were used in order to maintain mass of the split soleus muscle less than
25 40mg. The animals were fasted for 4 hours prior to sacrifice by decapitation. The skin was stripped from the lower limb which was then pinned out on corkboard. The tendo achilles was cut just above os calcis and m. gastrocnemius reflected out from the posterior aspect
30 of the tibia. M. soleus, a small 15-20mm long, 0.5mm thick flat muscle on the bone surface of m. gastrocnemius was then stripped clear and the perimysium cleaned off using fine scissors and forceps. M. soleus was then split
35 into equal parts using a blade passed antero-posteriorly through the belly of the muscle to obtain a total of 4 muscle strips from each animal. After dissecting the

muscle from the animal, it was kept for a short period in physiological saline. It was not necessary that the muscle be held under tension as this had no demonstrable effects on radioglucose incorporation into glycogen.

- 5 Muscles were added to 50mL Erlenmeyer flasks containing 10mL of a pregassed Krebs-Ringer bicarbonate buffer containing (each liter) NaCl 118.5 mmol (6.93g), KCl 5.94 mmol (443mg), CaCl_2 2.54 mmol (282mg), MgSO_4 1.19 mmol (143mg), KH_2PO_4 1.19 mmol (162mg), NaHCO_3 25 mmol
10 (2.1g), 5.5mmol glucose (1g) and recombinant human insulin (Humulin-R, Eli Lilly, IN) and the test compound, as detailed below. pH at 37°C was verified as being between 7.1 and 7.4. Muscles were assigned to different flasks so that the 4 muscle pieces from each animal were evenly
15 distributed among the different assay conditions. The incubation media were gassed by gently blowing carbogen (95% O_2 , 5% CO_2) over the surface while being continuously agitated at 37°C in an oscillating water bath. After a half-hour "preincubation" period, 0.5 μCi of U- ^{14}C -glucose
20 was added to each flask which was incubated for a further 60 minutes. Each muscle piece was then rapidly removed, blotted and frozen in liquid N_2 , weighed and stored for subsequent determination of ^{14}C -glycogen.

- ^{14}C -glycogen determination was performed in a 7mL
25 scintillation vial. Each frozen muscle specimen was placed in a vial and digested in 1mL 60% potassium hydroxide at 70°C for 45 minutes under continuous agitation. Dissolved glycogen was precipitated out onto the vial by the addition of 3mL absolute ethanol and
30 overnight cooling at -20°C. The supernatant was gently aspirated, the glycogen washed again with ethanol, aspirated and the precipitate dried under vacuum. All ethanol is evaporated to avoid quenching during scintillation counting. The remaining glycogen was
35 redissolved in 1mL water and 4mL scintillation fluid and counted for ^{14}C .

The rate of glucose incorporation into glycogen (expressed in $\mu\text{mol/g/hr}$) was obtained from the specific activity of ^{14}C -glucose in the 5.5mM glucose of the incubation medium, and the total ^{14}C counts remaining in the glycogen extracted from each muscle. Dose/response curves were fitted to a 4-parameter logistic model using a least-squares iterative routine (ALLFIT, v2.7, NIH, MD) to derive EC_{50} 's. Since EC_{50} is log-normally distributed, it is expressed \pm standard error of the logarithm. Pairwise comparisons were performed using t -test based routines of SYSTAT (Wilkinson, "SYSTAT: the system for statistics," SYSTAT Inc., Evanston IL (1989)).

Dose response curves were generated with muscles added to media containing 7.1nM (1000 $\mu\text{U/mL}$) insulin and each test compound added at final (nominal) concentrations of 0, 1, 3, 10, 30, 100, 300 and 1000nM. Each assay also contained internal positive controls consisting of a single batch of archived rat amylin, lyophilized and stored at -70°C .

Human amylin is a known hyperglycemic peptide, and EC_{50} measurements of amylin preparations in the soleus muscle assay range typically from about 1-10 nM, although some commercial preparations which are less than 90% pure have higher EC_{50} 's due to the presence of contaminants that result in a lower measured activity. Results for test compounds are set forth in Table I, showing that each of the compounds has amylin activity.

TABLE I

		Receptor Binding Assay <u>IC₅₀ (pM)</u>	Soleus Muscle Assay <u>EC₅₀ (nM)</u>
	1) ²⁸ Pro-h-Amylin	15.0	2.64
	2) ²⁵ Pro ²⁶ Val ^{38,29} Pro-h-Amylin	18.0	4.68
5	3) ^{2,7} Cyclo-[² Asp, ⁷ Lys]-h-Amylin	310.0	6.62
	4) ^{2,37} h-Amylin	236.0	1.63
	5) ¹ Ala-h-Amylin	148.0	12.78
	6) ¹ Ser-h-Amylin	33.0	8.70
	7) ²⁹ Pro-h-Amylin	64.0	3.75
10	8) ^{25,28} Pro-h-Amylin	26.0	13.20
	9) des- ¹ Lys ^{25,28} Pro-h-Amylin	85.0	7.70
	10) ¹⁸ Arg ^{25,28} Pro-h-Amylin	32.0	2.83
	11) des- ¹ Lys ¹⁸ Arg ^{25,28} Pro-h-Amylin	82.0	3.77
15	12) ¹⁸ Arg ^{25,28,29} Pro-h-Amylin	21.0	1.25
	13) des- ¹ Lys ¹⁸ Arg ^{25,28,29} Pro-h-Amylin	21.0	1.86
	14) ^{25,28,29} Pro-h-Amylin	10.0	3.71
	15) des- ¹ Lys ^{25,28,29} Pro-h-Amylin	14.0	4.15

TABLE II

	A ₁	B ₁	C ₁	D ₁	E ₁	E ₁	G ₁	H ₁	I ₁	I ₁	K ₁	L ₁	M ₁	Z ₁
18)	Lys	Ala	Val	His	Ser	Ser	Asn	Leu	Pro	Val	Pro	Pro	Asn	-NH ₂
19)	Lys	Ala	Val	His	Ser	Ser	Asn	Leu	Pro	Val	Pro	Ser	Asn	-NH ₂
5	Hydrogen	Ala	Val	His	Ser	Ser	Asn	Leu	Pro	Val	Pro	Ser	Asn	-NH ₂
21)	Lys	Ala	Val	Arg	Ser	Ser	Asn	Leu	Pro	Ile	Pro	Pro	Asn	-NH ₂
22)	Lys	Ala	Val	Arg	Ser	Ser	Asn	Leu	Pro	Ile	Pro	Ser	Asn	-NH ₂
23)	Lys	Ala	Val	Arg	Ser	Ser	Asn	Leu	Pro	Ile	Pro	Ser	Asn	-NH ₂
24)	Lys	Ala	Ile	His	Ser	Ser	Asn	Leu	Pro	Ile	Pro	Pro	Asn	-NH ₂
25)	Lys	Ala	Ile	His	Ser	Ser	Asn	Phe	Pro	Ile	Pro	Pro	Asn	-NH ₂
10	Hydrogen	Ala	Ile	His	Ser	Ser	Asn	Leu	Pro	Ile	Pro	Pro	Asn	-NH ₂
27)	Lys	Ala	Ile	Arg	Ser	Ser	Asn	Leu	Ala	Ile	Ser	Ser	Asn	-NH ₂
28)	Lys	Ala	Ile	Arg	Ser	Ser	Asn	Leu	Ala	Val	Pro	Ser	Asn	-NH ₂
29)	Lys	Ala	Ile	Arg	Ser	Ser	Asn	Leu	Pro	Val	Pro	Pro	Asn	-NH ₂
30)	Lys	Thr	Val	His	Ser	Ser	His	Leu	Ala	Ala	Leu	Pro	Asp	-NH ₂
15	Lys	Thr	Val	His	Ser	Ser	His	Leu	Ala	Ala	Leu	Pro	Asp	-NH ₂
31)	Lys	Thr	Val	His	Ser	Ser	His	Leu	Ala	Ala	Leu	Pro	Asp	-NH ₂
32)	Hydrogen	Thr	Val	His	Ser	Ser	His	Leu	Ala	Ala	Leu	Pro	Asp	-NH ₂
33)	Lys	Thr	Val	Arg	Ser	Ser	His	Leu	Ala	Ala	Ser	Pro	Asp	-NH ₂
34)	Lys	Thr	Val	Arg	Ser	Ser	His	Leu	Ala	Ile	Pro	Pro	Asp	-NH ₂
20	Lys	Thr	Val	Arg	Ser	Ser	His	Leu	Pro	Ala	Pro	Pro	Asp	-NH ₂

Claims

1. An agonist analogue of amylin having the amino acid sequence depicted in Figure 3 wherein

- A₁ is Lys, Ala, Ser or hydrogen,
5 B₁ is Ala, Ser or Thr;
C₁ is Val, Leu or Ile;
D₁ is His or Arg;
E₁ is Ser or Thr;
F₁ is Ser, Thr, Gln or Asn;
10 G₁ is Asn, Gln or His;
H₁ is Phe, Leu or Tyr;
I₁ is Ala or Pro;
J₁ is Ile, Val, Ala or Leu;
K₁ is Ser, Pro, Leu, Ile or Thr;
15 J₁ is Ser, Pro or Thr;
M₁ is Asn, Asp, Gln or Asn; X and Y are independently selected residues having side chains which are chemically bonded to each other to form an intramolecular linkage; and Z is amino, alkylamino, dialkylamino, cycloalkylamino, 20 arylamino, aralkylamino, alkyloxy, aryloxy or aralkyloxy; and provided that when
- (a) A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is His, E₁ is Ser, F₁ is Ser, G₁ is Asn, H₁ is Phe, I₁ is Ala, J₁ is Ile, K₁ is Ser, L₁ is Ser, and M₁ is Asn;
25 (b) A₁ is Lys, B₁ is Ala, C₁ is Ile, D₁ is Arg, E₁ is Ser, F₁ is Ser, G₁ is Asn, H₁ is Leu, I₁ is Ala, J₁ is Ile, K₁ is Ser, L₁ is Pro, and M₁ is Asn;
(c) A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is Arg, E₁ is Thr, F₁ is Ser, G₁ is Asn, H₁ is Leu, I₁ is Ala, J₁ is Ile, K₁ is Ser, L₁ is Pro, and M₁ is Asn;
30 (d) A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is Arg, E₁ is Ser, F₁ is Ser, G₁ is Asn, H₁ is Leu, I₁ is Pro, J₁ is Val, K₁ is Pro, L₁ is Pro, and M₁ is Asn;
(e) A₁ is Lys, B₁ is Ala, C₁ is Val, D₁ is His, E₁ is Ser, F₁ is Asn, G₁ is Asn, H₁ is Leu, I₁ is Pro, J₁ is Val, K₁ is Ser, L₁ is Pro, and M₁ is Asn; or
35

(f) A₁ is Lys, B₁ is Thr, C₁ is Val, D₁ is Arg, E₁ is Ser, F₁ is Ser, G₁ is His, H₁ is Leu, I₁ is Ala, J₁ is Ala, K₁ is Leu, L₁ is Pro, and M₁ is Asp;
then one or more of any of A₁ to M₁ is not an L-
5 amino acid and Z is not amino.

2. An agonist analogue of amylin according to claim 1 wherein X and Y have side chains selected to form an intramolecular linkage which comprises a disulfide bond, a lactam, an alkylene linkage, an alkenyl linkage, an
10 alkynyl linkage, an ether linkage or a thioether linkage.

3. An agonist analogue of amylin according to claim 2 wherein X and Y comprise Cys residues linked by a disulfide bond.

4. An agonist analogue of amylin according to claim
15 3 wherein at least one of I₁, K₁ and L₁ is Pro.

5. An agonist analogue of amylin according to claim 4 wherein two of I₁, K₁ and L₁ are Pro.

6. An agonist analogue of amylin according to claim 4 wherein I₁, K₁ and L₁ are Pro.

20 7. An agonist analogue of amylin according to claim 4, 5 or 6 wherein D₁ is Arg.

8. An agonist analogue of amylin according to claim 4, 5 or 6 wherein J₁ is Val.

9. An agonist analogue of amylin according to claim
25 7 wherein A₁ is Lys.

10. An agonist analogue of amylin according to claim 7 wherein A₁ is hydrogen.

11. An agonist analogue of amylin according to claim
8 wherein A₁ is Lys.
12. An agonist analogue of amylin according to claim
1 wherein said intramolecular linkage comprises a lactam,
5 an alkylene, alkenyl, alkynyl, ether or thioether linkage.
13. An agonist analogue of amylin according to claim
12 wherein at least one of I₁, K₁ and L₁ is Pro.
14. An agonist analogue of amylin according to claim
13 wherein at least two of I₁, K₁ and L₁ are Pro.
- 10 15. An agonist analogue of amylin according to claim
13 wherein I₁, K₁ and L₁ is Pro.
16. An agonist analogue of amylin according to claim
13, 14 or 15 wherein D₁ is Arg.
17. An agonist analogue of amylin according to claim
15 16 wherein H₁ is Leu.
18. An agonist analogue of amylin according to claim
17 wherein A₁ is Lys.
19. An agonist analogue of amylin according to claim
16 wherein A₁ is Lys.
- 20 20. An agonist analogue of amylin according to claim
16 wherein A₁ is hydrogen.
21. An agonist analogue of amylin having the amino
acid sequence depicted in Figure 3 which is not bridged
and wherein
25 A₁ is Lys, Ala, Ser or hydrogen, des- α -amino Lys
or acetylated Lys;
B₁ is Ala, Ser or Thr;

33

- C₁ is Val, Leu or Ile;
D₁ is His or Arg;
E₁ is Ser or Thr;
F₁ is Ser, Thr, Gln or Asn;
5 G₁ is Asn, Gln or His;
H₁ is Phe, Leu or Tyr;
I₁ is Ala or Pro;
J₁ is Ile, Val, Ala or Leu;
K₁ is Ser, Pro, Leu, Ile or Thr;
10 L₁ is Ser, Pro or Thr;
M₁ is Asn, Asp, Gln or Asn;
- wherein X and Y are independently Ala, Ser, Cys, Val, Leu, Ile or an alkyl, aryl or aralkyl ester of Ser or Cys; and Z is amino, alkylamino, dialkylamino, cycloalkylamino, 15 arylamino, aralkylamino, alkylloxy, aryloxy or aralkyloxy.

22. A method for the treatment of diabetes mellitus and symptoms thereof comprising the administration of a therapeutically effective amount of an agonist analogue of amylin according to claim 1.

- 20 23. A method for the treatment of diabetes mellitus and symptoms thereof comprising the administration of a therapeutically effective amount of an agonist analogue of amylin according to claim 3.

24. A method for the treatment of diabetes mellitus 25 and symptoms thereof comprising the administration of a therapeutically effective amount of an agonist analogue of amylin according to claim 21.

25. The method of claim 22 further comprising the administration of a therapeutically effective amount of an 30 insulin.

26. The method of claim 23 further comprising the administration of a therapeutically effective amount of an insulin.

27. The method of claim 24 further comprising the
5 administration of a therapeutically effective amount of an insulin.

28. A method for the treatment of a hypoglycemic condition in a mammal comprising the step of administering a therapeutically effective amount of an agonist analogue
10 of amylin according to claim 1.

29. A method for the treatment of a hypoglycemic condition in a mammal comprising the step of administering a therapeutically effective amount of an agonist analogue of amylin according to claim 3.

30. A method for the treatment of a hypoglycemic condition in a mammal comprising the step of administering a therapeutically effective amount of an agonist analogue of amylin according to claim 21.
15

31. The method of claim 28 further comprising the
20 administration of a therapeutically effective amount of a glucagon.

32. The method of claim 29 further comprising the administration of a therapeutically effective amount of a glucagon.

33. The method of claim 30 further comprising the
25 administration of a therapeutically effective amount of a glucagon.

34. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 1.

35. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 3.

36. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 21.

37. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 1 and an insulin admixed in a form suitable for therapeutic administration.

38. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 3 and an insulin admixed in a form suitable for therapeutic administration.

39. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 21 and an insulin admixed in a form suitable for therapeutic administration.

40. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 1 and a glucagon admixed in a form suitable for therapeutic administration.

41. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 3 and a glucagon admixed in a form suitable for therapeutic administration.

42. A composition comprising a therapeutically effective amount of an agonist analogue of amylin according to claim 21 and a glucagon admixed in a form suitable for therapeutic administration.

5 43. The composition of any of claims 34, 37 or 40 wherein said agonist analogue of amylin is ^{25,28,29}Pro-h-amylin.

44. The composition of any of claims 34, 37 or 40 wherein said agonist analogue of amylin is des-¹Lys^{25,28,29}
10 Pro-h-amylin.

45. The composition of any of claims 34, 37 or 40 wherein said agonist analogue of amylin is ¹⁸Arg^{25,28}Pro-h-amylin.

46. The composition of any of claims 34, 37 or 40
15 wherein said agonist analogue of amylin is des-¹Lys¹⁸Arg^{25,28}Pro-h-amylin.

FIG. 1.

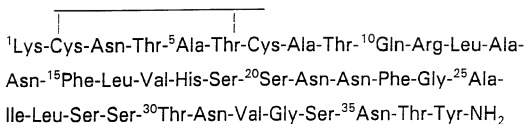


FIG. 2.

Amylin

human

KdCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH₂

cat -----IR---L---P-----

dog -----RT---L---P-----

rat -----R---L-PV-PP-----

mouse -----R---L-PV-PP-----

hamster -----N--L-PV--P-----

guinea pig -----T---R-H-L--A-LP-D-----

FIG. 3.

¹A₁-X-Asn-Thr-⁵Ala-Thr-Y-Ala-Thr-¹⁰Gln-Arg-Leu-
B₁-Asn-¹⁵Phe-Leu-C₁-D₁-E₁-²⁰F₁-G₁-Asn-H₁-Gly-²⁵I₁-J₁-
Leu-K₁-L₁-³⁰Thr-M₁-Val-Gly-Ser-³⁵Asn-Thr-Tyr-Z

I. CLASSIFICATION OF SUBJECT MATTER (if several classifications symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07K7/10; A61K37/02		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	WO,A,9 211 862 (AMYLIN PHARMCEUTICALS INC.) 23 July 1992 see page 5, line 21 - line 24 see page 13, line 28 - page 14, line 37 see page 19, line 15 - page 20, line 20; claims 1-5; example 4 ---	1-4, 12, 13, 22-24, 28,29, 31,32, 34,35, 40,41
P,X	WO,A,9 215 317 (AMYLIN PHARMACEUTICALS, INC.) 17 September 1992 cited in the application see page 14, paragraph 2 - page 15, paragraph 1; claims 28,29,32,33; examples --- -/-	1-4
⁹ Special categories of cited documents: ¹⁰ ^{"A"} document defining the general state of the art which is not considered to be of particular relevance ^{"E"} earlier document but published on or after the international filing date ^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) ^{"O"} document referring to an oral disclosure, use, exhibition or other means ^{"P"} document published prior to the international filing date but later than the priority date claimed ^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention ^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step ^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art ^{"&"} document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
17 MARCH 1993	29. 03. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	FUHR C.K.B.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
X	EP,A,0 408 294 (AMYLIN CORPORATION) 16 January 1991 see page 7, line 4 - line 22; claims 6,8,10,11 ---	21
X	WO,A,8 906 135 (AMYLIN CORPORATION) 13 July 1989 see claims 1-4 ---	21
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 86, no. 15, August 1989, WASHINGTON US pages 5738 - 5742 M. NICHII ET AL 'Conservation of the sequence of islet amyloid polypeptide in five mammals is consistent with its putative role as an islet hormone' see abstract ---	1
A	EP,A,0 309 100 (AMYLIN CORPORATION) 29 March 1989 see page 3, line 51 - page 4, line 18; claims 1-12; example -----	22,23, 25,26, 37,38

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/09842

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 22-33 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9209842
SA 67027

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 17/03/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9211862	23-07-92	AU-A- 1328692	17-08-92
		EP-A- 0525149	03-02-93
		EP-A- 0525158	03-02-93
		WO-A- 9211863	23-07-92
WO-A-9215317	17-09-92	None	
EP-A-0408294	16-01-91	AU-B- 620727	20-02-92
		AU-A- 5953790	06-02-91
		CA-A- 2020786	11-01-91
		JP-T- 4500688	06-02-92
		WO-A- 9100737	24-01-91
WO-A-8906135	13-07-89	AU-A- 2949489	01-08-89
		EP-A- 0348490	03-01-90
		JP-T- 3501611	11-04-91
EP-A-0309100	29-03-89	AU-A- 2157588	20-07-89
		JP-A- 1096137	14-04-89
		US-A- 5124314	23-06-92